Cryopreservation of Human Spermatozoa : Comparison of Two Cryoprotectants and Two Freezing Methods

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Abstract

A prospective experimental study was performed to compare sperm cryosurvival rate between two kinds of cryoprotective media: citrate-egg yolk-glycerol (CEG) and TEST-citrate-egg yolk-glycerol (TEST-EYG), and between two freezing methods : noncontrolled rate (NCR) freezing and computer controlled rate (CR) freezing. One hundred semen samples were included in the study. Each sample was divided into two parts, which were prepared as two CEG and two TEST-EYG mixture straws, then one straw from each mixture was frozen by the NCR-freezing method and the complementary straw was frozen by the CR-freezing method. After one week of cryostorage, the straws were thawed and the post-thaw sperm survival was assessed. As the results, the post-thaw sperm survival rate of the CEG media group was slightly higher than the TEST-EYG group in both the NCR-freezing method (51.2% *vs* 47.0%, $p < 0.05$) and the CR-freezing method (48.2% *vs* 43.4%, $p < 0.05$); and similarly; the NCR-freezing group yielded a slightly higher post-thaw sperm survival rate than the CR-freezing group in both the CEG media used (51.2% *vs* 48.2%, $p < 0.05$) and the TEST-EYG media used (47.0% *vs* 43.4%, $p < 0.05$). However, these did not have clinical significance because the differences were too small. In conclusion, the use of either CEG or TEST-EYG media, and either the NCR or CR-freezing method did not have clinical differences on the post-thaw sperm survival.

Cryopreservation of human spermatozoa is an essential technique involved in male infertility treatment. Since the risk of human immuno deficiency virus (HIV) transmission from fresh donor semen is concerned, the American Fertility Society (AFS, 1988)[1] now recommends the use only of seronegative donor semen, after at least 6 months cryostorage, for artificial donor insemination. Thus, the establishment of a simple and effective cryopreservative technique that yields high post-thaw sperm survival rate is essential for the sperm bank service.

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The success of human sperm cryopreservation can be influenced by several factors including the nature of the cryoprotective media used for freezing. Glycerol was the cryoprotectant used in the first successful human sperm freezing, reported by Sherman in 1953[2], but it was demonstrated to have a toxic effect on spermatozoa, especially at room temperature[3]. A more complex media composed of citrate-egg yolk-glycerol (CEG) has been reported to result in a higher pregnancy rate compared to glycerol alone[4-6] and has been used extensively in many sperm banks. Graham (1972)[7] developed a zwitterion buffer system composed of n-tris (hydroxymethyl) methyl - 2 - aminoethane sulfonic acid (TES) and tris (hydroxy methyl) aminoethane (Tris) in addition to egg yolk and glycerol (TEST-EYG) and noted a good result in bovine sperm freezing. Weidel and Prins[8] later confirmed a better post - thaw sperm outcome using TEST-EYG compared with the CEG media in a pooled human semen study, however, their result was not supported by Wei-Jie[9].

The method of freezing is another factor that may have an effect on sperm cryosurvival. Most studies reported that slow cooling rate method is superior to rapid cooling[10,11]; but the advantage of computer controlled rate (CR) freezing over noncontrolled rate (NCR) freezing is still controversial. Sarafini and Marrs[12] reported a significantly better post-thaw sperm outcome when computer controlled rate (CR) freezing was used compared to noncontrolled rate (NCR) freezing which cooled in liquid nitrogen vapor. However, the studies of Thalchil[13] and Wei-Jie[9] did not confirm the different outcome of these two methods. The purpose of this study was, therefore, to compare the post-thaw human sperm survival rate between the use of the citrate-egg yolk-glycerol (CEG) and the TEST-citrate-egg yolk-glycerol (TEST-EYG) as cryoprotective media; and between the computer controlled rate (CR) and noncontrolled rate (NCR) as the freezing method.

MATERIAL AND METHOD

This study was performed as a prospective experimental trial.

Semen samples

The donor volunteers were asked to produce semen samples collected in sterile containers. After 30 minutes of liquefaction, a small amount of the semen was taken for standard semen analysis. Only the semen samples that had count > 20 x 10^6/ml, motility > 50 per cent, normal morphology > 30 per cent, and white blood cell < 1 cell/ml were included into the study.

Preparation of the cryoprotective media

Citrate-egg yolk-glycerol media (CEG) composed of : 20 per cent v/v egg yolk, 15 per cent v/v glycerol, 26 per cent v/v of 5.5 per cent glucose in water, and 39 per cent v/v of 3 per cent sodium citrate in water.

Test-citrate-egg yolk-glycerol media (TEST-EYG) composed of: 56 per cent TEST, 4 per cent fructose, 20 per cent citrate solution, 20 per cent egg yolk and added glycerol to make final concentration to 21 per cent v/v.

Equilibration with cryoprotective media

Each semen sample was divided into two parts. The first part was mixed with an equal volume of citrate-egg yolk-glycerol (CEG) media by adding the media dropwise into the semen whilst swirling for 10-15 minutes, the second part was mixed, in a similar way, with the TEST-citrate-egg yolk-glycerol (TEST-EYG). Each semen mixture was aspirated into two 2.5 ml straws, sealed at both ends and labelled with a marker.

Noncontrolled rate (NCR) freezing method

One straw from each CEG semen mixture and one straw from each TEST-EYG semen mixture were placed in the ultracold refrigerator at -80°C for 1 hour, they were then transferred into liquid nitrogen and stored for one week.

Computer controlled rate (CR) freezing method

The complementary CEG and TEST-EYG straws of the NCR-freezing method were placed in the freezing chamber of the computer controlled rate freezing machine (PLANER BIOMED, KRYO 10 SERIES II). The temperature was then reduced stepwise as follow : 1°C/min from room temperature to -30°C, and 5°C/min from -30°C to -80°C, then quickly transferred into liquid introgen and stored likewise for one week.

Thawing

After one week of cryostorage, the straws were thawed by removal from the liquid nitrogen and placed in room temperature for 20 minutes.

The post-thaw sperm motility was assessed. The cryosurvival rate was calculated as follows:

$$\text{percentage of cryosurvival} = \frac{\text{post-thaw sperm motility x 100}}{\text{pre-freeze sperm motility}}$$

Statistical analysis

The data was analysed using the paired *t*-test to compare the post-thaw sperm motility and survival rate between each group frozen by the CEG or TEST-EYG cryoprotectant, and the NCR or CR freezing method. These were performed by SPSS computer software.

Table 1. Fresh semen characteristics before freezing (n = 100).

Variables	Average value
Volume (ml)	2.2 (0.5-5.2)
Sperm count (x 10^6)	69 (32-182)
Normal morphology (%)	65 (50-87)
Motility (%)	68 (53-85)
grade a*(%)	42 (9-67)
grade b*(%)	17 (0-48)
grade c*(%)	9 (9-24)

* grade a: rapid progressive motility
grade b: slow progressive motility
grade c: non progressive motility

RESULTS

One hundred semen samples were included in the study. The mean age of the donor volunteers was 32 years old. The average characteristics of the fresh semen before freezing were as follows: volume 2.2 ml, sperm count 69 x 10^6/ml, normal morphology 65 per cent, motility 68 per cent, (rapid progressive 42%, slow progressive 17%, nonprogressive 9%) (Table 1).

After one week of cryostorage, all the four different freezing techniques resulted in reduced sperm motility, especially the percentage of rapid progressive sperm. The average post-thaw sperm motility for the CEG-NGR, CEG-CR, TEST-EYG-NCR, and TEST-EYG-CR freezing techniques were 35.9 per cent, 32.9 per cent, 32.3 per cent, and 29.8 per cent respectively (Fig. 1); and the average percentage of cryosurvival rate was 51.2 per cent, 48.2 per cent, 47.0 per cent, and 43.4 per cent respectively (Fig. 2).

In comparison between the two cryopreservative media, the use of the CEG media yielded a slightly higher percentage of post-thaw sperm motility and survival rate than the TEST-EYG media in both the NCR-freezing group (motility 35.1% *vs* 32.3%, survival 51.2% *vs* 47.0%, $p < 0.05$) and the CR-freezing group (motility 32.9% *vs* 28.8%, survival 48.2%, *vs* 43.4%, $p < 0.05$) (Table 2), but the differences were too small to have clinical significance. In comparison between the two freezing methods, the NCR-freezing group

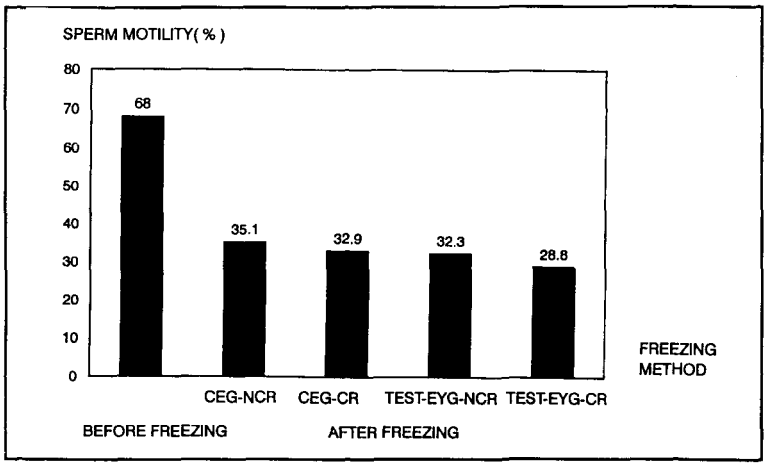

Fig. 1. The average post-thaw sperm motility of the four freezing methods : CEG-NCR, CEG-CR, TEST-EYG-NCR, TEST-EYG-CR, * compare to the pre-freeze.

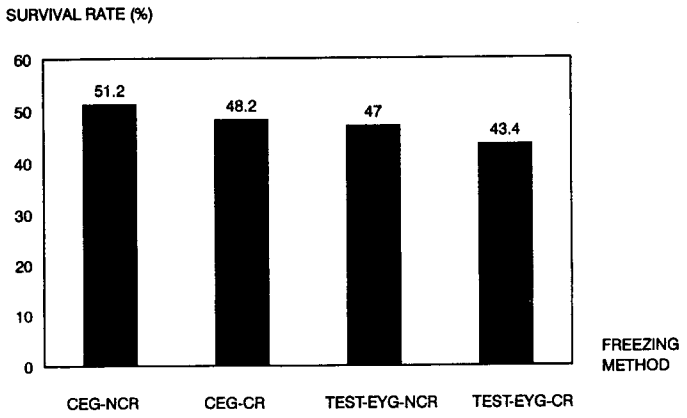

Fig. 2. The average post-thaw survial rate of the four freezing methods: CEG-NCR, CEG-CR, TEST-EYG-NCR, TEST-EYG-CR*.

*** CEG-NCR: use citrate egg yolk-glycerol as cryopreservative media, and cooled by noncontrolled rate freezing.**
CEG-CR : use citrate-egg yolk-glycerol as cryopreservative media, and cooled by controlled rate freezing.
TESY-EYG-NCR : use TEST-citrate-egg yolk-glycerol as cryopreservative media, and cooled by noncontrolled rate freezing.
TEST-EYG-CR : use TEST-citrate-egg yolk-glycerol as cryopreservative media, and cooled by controlled rate freezing.

Table 2. Comparison of the post-thaw sperm motility and survival rate between the two cryoprotectants: CEG and TEST-EYG media (n = 100).

Method of freezing	CEG (mean)	TEST-EYG (mean)	95% confident interval of difference (mean difference + 1.96 SE)	p-value
Noncontrolled rate freezing (NCR)				
Sperm motility (%)	35.1	32.3	(1.2, 4.4)	0.001*
grade a (%)	13.7	11.2	(0.8, 4.2)	0.005*
grade b (%)	9.7	8.9	(-0.4, 2.0)	0.197
grade c (%)	11.7	12.2	(-1.7, 0.7)	0.386
Survival rate (%)	51.2	47.0	(1.7, 6.5)	0.008*
Computer controlled rate freezing (CR)				
Sperm motility (%)	32.9	28.8	(1.3, 4.9)	0.001*
grade a (%)	11.0	9.0	(0.2, 3.8)	0.036*
grade b (%)	9.0	7.9	(-0.1, 2.3)	0.090
grade c (%)	13.2	12.7	(-0.9, 1.9)	0.529
Survival rate (%)	48.2	43.4	(2.1, 7.5)	0.0006*

* $p < 0.05$

Table 3. Comparison of the post-thaw sperm motility and survival rate between the two freezing method: noncontrolled rate (NCR) and computer controlled rate (CR) (n = 100).

Cryoprotectant	NCR-freezing (mean)	CR-freezing (mean)	95% confident interval of difference (mean difference + 1.96 SE)	p-value
CEG media				
Sperm motility (%)	35.1	32.9	(0.4, 3.8)	0.017*
grade a (%)	13.7	11.0	(1.1, 4.3)	0.001*
grade b (%)	9.7	9.0	(-0.6, 2.0)	0.309
grade c (%)	11.7	13.2	(-2.1, -0.9)	0.014*
Survival rate (%)	51.2	48.2	(0.4, 5.4)	0.023*
TEST-EYG media				
Sperm motility (%)	32.3	29.8	(0.8, 4.2)	0.006*
grade a (%)	11.2	9.0	(0.5, 3.7)	0.012*
grade b (%)	8.9	7.9	(-0.2, 2.2)	0.123
grade c (%)	12.2	12.7	(-1.8, 0.6)	0.356
Survival rate (%)	47.0	43.4	(1.1, 6.3)	0.006*

* $p < 0.05$

yielded a slightly higher percentage of post-thaw motility and survival rate than the CR-freezing group in both the CEG media used (motility 35.1% *vs* 32.9%, survival 51.2% *vs* 48.2%, $p < 0.05$) and the TEST-EYG media used (motility 32.3% *vs* 29.8%, survival 47.0% *vs* 43.4%, $p < 0.05$) (Table 3), again, the differences did not have clinical significance.

DISCUSSION

The basic principal of cryopreservation is that the cell is equilibrated with cryoprotectant, cooled to subzero temperature, kept cryostorage in liquid nitrogen until use, then thawed and finally the cryoprotectant is removed. To obtain a high survival rate, the cell needs to maintain its structural integrity throughout the freezing and thawing process. The degree of cellular injury is determined by the total volume of ice crystal formation in the cell. Cryoprotectant is capable of forming hydrogen bonds with water molecules to prevent lethal intracellular ice formation[14]. During the cooling process, ice will form first in the extracellular fluid which makes the solute become gradually more concentrated. As the extracellular osmolarity increases, the water will be drawn out of the cell because the ice crystal cannot move across the cell membrane. The rate of cooling has to be slow enough to prevent intracellular ice formation, but not too slow to cause cell damage from excessive dehydration and high salt concentration[15]. Thus, the nature of cryoprotectant and the method of freezing are major factors that can influence the cryosurvival.

The first objective of this study was to compare the cryoprotective effect between the CEG and the TEST-EYG media. The CEG media contains eggyolk which has a stabilizing effect preventing the sperm membrane from being damaged by free oxygen radicals[16], and citrate which takes part in the bicarbonate-citrate buffer system in semen[17]. The zwitterion TES-Tris buffer included in the TEST-EYG media is related to the dipolar ion's capacity to bind with the free hydrogen and hydroxyl ion in the surrounding media, thereby aiding the dehydration process[8]. However, this study did not demonstrate an advantage effect of TEST-EYG media over the CEG media in either the NCR-freezing group or the CR-freezing group, as reported by Weidel and Prins[8].

For the second objective of the study to compare the non controlled rate (NCR) and computer controlled rate (CR) freezing, the latter technique is more expensive and time-consuming, but may have advantages due to more precise and reproducible cooling rate, which should lead to more effective, uniform dehydration and prevention of intracellular ice formation[18]. However, we did not observe a better result of the CR-freezing over

the NCR-freezing in both the CEG and the TEST-EYG cryoprotectant groups. This outcome was similar to those of Thachil[13] and Wei-Jie[9]. One explanation may be that human spermatozoa is relatively insensitive to the phenomenon of temperature shock in terms of motility and survival recovery. This is possibly because of the low water content in spermatozoa which mostly consists of extremely condensed DNA in the nucleus[17].

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การแช่แข็งเชื้ออสุจิ : เปรียบเทียบระหว่างครัยโอโปรเท็คแท๊นท์ 2 ชนิด และเทคนิคการแช่แข็ง 2 วิธี

สุภักดี จุลวิจิตรพงษ์, พ.บ., สมบูรณ์ คุณาธิคม, พ.บ.*,*
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รายงานการศึกษาเปรียบเทียบการแช่แข็งเชื้ออสุจิ ระหว่างการใช้ Cryoprotective media 2 ชนิด คือ Citrate-eggyolk-glycerol (CEG) กับ TEST-citrate-eggyolk-glycerol (TEST-EYG) และเทคนิคการแช่แข็ง 2 วิธี คือ วิธีไม่ควบคุมอัตราการลดของอุณหภูมิ (Noncontrolled rate freezing : NCR) กับวิธีควบคุมอัตราการลดของอุณหภูมิด้วยเครื่องคอมพิวเตอร์ (Computer controlled rate freezing : CR) จำนวนเชื้ออสุจิที่ทำการศึกษา 100 ตัวอย่าง แต่ละตัวอย่างถูกแบ่งออกเป็น 2 ส่วน เพื่อผสมกับ Cryoprotectant แต่ละชนิด ดูดส่วนผสมเชื้ออสุจิแต่ละชนิดเข้าในหลอดเก็บเชื้ออสุจิ (straw) ส่วนผสมละ 2 หลอด ทำการแช่แข็งหลอดเก็บเชื้ออสุจิหลอดแรกของแต่ละส่วนผสมด้วยวิธีไม่ควบคุมอัตราการลดของอุณหภูมิ (NCR) และหลอดที่สองด้วยวิธีควบคุมอัตราการลดของอุณหภูมิโดยคอมพิวเตอร์ (CR) ภายหลังการเก็บแช่แข็งไว้นาน 1 สัปดาห์ จึงนำมาวิเคราะห์หาอัตราการอยู่รอดของเชื้ออสุจิ ผลการศึกษาพบว่าอัตราการอยู่รอดของเชื้ออสุจิในกลุ่มที่ใช้ CEG เป็น cryoprotectant สูงกว่ากลุ่มที่ใช้ TEST-EYG เล็กน้อย และเช่นเดียวกัน กลุ่มที่ทำการแช่แข็งด้วยวิธี NCR มีอัตราการอยู่รอดของเชื้ออสุจิสูงกว่าวิธี CR เล็กน้อย ซึ่งความแตกต่างนี้ไม่มีนัยสำคัญทางคลินิก สรุปว่า cryoprotectant ทั้งสองชนิด และเทคนิคการแช่แข็งทั้งสองวิธี ไม่มีผลแตกต่างกันทางคลินิกต่ออัตราการอยู่รอดของเชื้ออสุจิภายหลังการเก็บแช่แข็ง

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